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Review

Common Themes in the Design and Function of Bacterial Effectors

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Central to the biology of many pathogenic bacteria are a number of specialized machines, known as type III, type IV, or type VI protein secretion systems. These machines have specifically evolved to deliver bacterial effector proteins into host cells with the capacity to modulate a variety of cellular functions. The identification of the biochemical activities of many effector proteins, coupled with a better understanding of their potential contribution to pathogenesis, has revealed common themes in the evolutionary design and function of these remarkable bacterial proteins.

Introduction

Many gram-negative bacteria that are pathogenic or symbiotic for plants, insects, or animals have evolved complex machines to transfer multiple bacterially encoded proteins into eukaryotic cells. At least three different types of these machines, which for historical reasons are referred to as type III, type IV, and type VI protein secretion systems, have been described (Christie et al., 2005; Filloux et al., 2008; Galán and Wolf-Watz, 2006). A great deal of attention has been devoted to the study of these machines, because they are central to the pathogenesis of the bacterial pathogens that encode them. Proteins delivered by these machines have the capacity to modulate a variety of cellular functions and are collectively known as effectors. Effectors are distinct from bacterial toxins, which are also bacterial products that exert their function on living cells or organisms (Alouf, 2000). A unique property of toxins is that their toxic effects can be observed when exogenously added to living organisms or cells. In contrast, the term "effectors" should be reserved for molecules that require specialized multiprotein machines for their direct delivery into target cells. However, the distinction between toxins and effectors reflects more than just differences in their delivery mechanisms. Bacterial toxins usually have a single biochemical activity that directly exerts its effect on specific cellular targets. By contrast, effector proteins exert their specific function in concert with the activities of multiple other bacterial effectors delivered by the same machine. The activities of effector proteins are often subtle and more "tuned" to modulate cellular functions rather than to irreversibly disrupt cellular homeostasis. In fact, the evolution of protein delivery machines such as type III, type IV, and type VI secretion systems may have been specifically driven by the need to deliver multiple proteins in a coordinated fashion to modulate complex cellular processes.

The study of effector proteins delivered by these specialized machines has provided remarkable insight not only into fundamental aspects of host/pathogen interactions, but also into the basic biology of eukaryotic cells. Despite the diversity of activities associated with the different effector proteins, it is now evident that there are a number of common themes that characterize the function of these molecules. The common themes in the evolutionary design of these molecules will be the subject of this review. In addition, the significant challenges to the study of these effector proteins stemming from their unique properties will also be discussed. In discussing these general themes, I will be drawing from specific examples of effectors delivered by type III secretion systems (T3SSs), since they are the best characterized. However, I believe that these principles are applicable to bacterial effector proteins delivered by other machines. The purpose of this article is not to comprehensively review the literature of T3SSs or bacterial effector proteins. Rather, the intent is to discuss a limited set of specific examples of T3SS effectors that best illustrate the existence of common principles in the design and function of bacterial effector proteins.

Mimicry of Host Cell Proteins

T3SSs are encoded by many important pathogenic bacteria, including *Salmonella enterica* serovars (e.g., *S.* Typhimurium, *S.* Typhi, etc.), *Shigella* spp., *Yersinia* spp., *Chlamydia* spp., *Pseudomonas* spp., *Vibrio* spp., *Bordetella* spp., and pathogenic strains of *E. coli* (Galán and Wolf-Watz, 2006). All of these bacteria have evolved complex and unique functional interfaces with eukaryotic cells, and their T3SSs are central components of these interfaces. A theme that has emerged over the last few years is that many T3SS effector proteins exert their function by mimicking activities of endogenous cellular proteins (Stebbins and Galán, 2001). Such mimicry can sometimes be detected at the level of the primary amino acid sequence. For example, some effectors share significant amino acid sequence similarity to eukaryotic cell proteins (e.g., protein kinases or phosphatases) (Galyov et al., 1993; Guan and Dixon, 1990). Therefore, their potential biochemical activities can be simply predicted from primary amino acid sequence analysis, although the identification of the cellular targets of these activities most often requires specific experimentation. However, this straightforward mimicry is rarely observed among most bacterial effectors. In fact, many effectors faithfully mimic the activities of cellular proteins without detectable amino acid sequence similarity (Stebbins and Galán, 2001). In such cases, the activities of the effector proteins cannot be predicted from their amino acid sequence analysis or even from their *proto* atomic structures (i.e., the atomic structures of the effector proteins by themselves and not in complex with their targets). For example, some

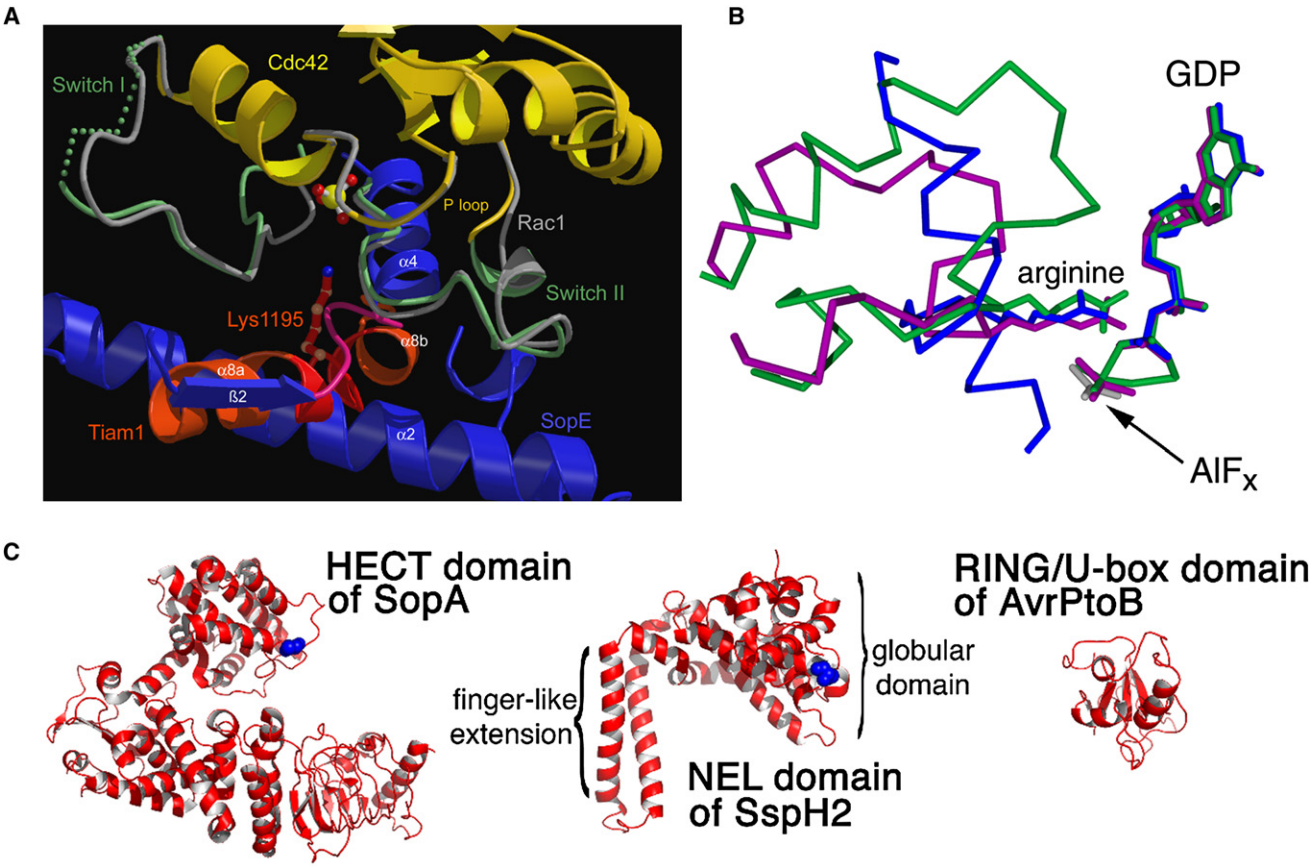

Figure 1. Structural Mimicry in Type III Secreted Effector Proteins

(A) *S.* Typhimurium SopE mimics Rho family GEFs. The conformational changes induced on the G domain of Cdc42 by SopE (shown in green) are similar to those induced by mammalian exchange factor Tiam1 on Rac1 (shown in gray). The P loop, switch I/II regions are shown for the individual molecules. Relevant regions of SopE and Tiam1 are shown in red and blue, respectively. The view shows the similar location of the catalytic loop from SopE and the important Lys1195 from Tiam (taken from Buchwald et al., 2002).

(B) *S.* Typhimurium SptP mimics Rho family GAPs. The active sites of three transition-state complexes between small GTPases (Rac1, Cdc42, and Ras) and their cognate GAPs (SptP, Cdc42 GAP, and Ras GAP) depicting the nucleotide and catalytic arginine present in all known GAPs are shown. This image illustrates that despite using a similar chemistry to the host factors, SptP (in blue) presents the arginine from a completely different protein architecture. AlFX, aluminum fluoride (taken from Stebbins and Galán, 2001).

(C) Effector proteins mimic three different types of E3 ligases. Shown are the E3 ligase domains of *S.* Typhimurium SspH2, showing the NEL domain; *S.* Typhimurium SopA, a HECT family of cysteine-dependent E3 ubiquitin ligases from *Salmonella*; and AvrPtoB, a RING finger/U-box protein. The catalytic cysteine residues are shown in a space-filling format colored blue (taken from Quezada et al., 2009).

effector proteins target Rho family GTPases by mimicking the activities of guanine nucleotide exchange factors (GEFs) or GTPase-activating proteins (GAPs). Yet there is nothing in their primary amino acid sequence that would suggest these activities. Such is the case for the *S.* Typhimurium T3SS effectors SopE and SptP, which are a GEF and a GAP, respectively, for Rac, Cdc42, and RhoG (Fu and Galán, 1999; Hardt et al., 1998a). The crystal structure of the effector/target protein complex shows conformational changes imposed by SopE on the nucleotide-binding region of Rac1 that are virtually identical to those imposed by an endogenous exchange factor such as Tiam1 (Figure 1A) (Buchwald et al., 2002). However, to introduce those conformational changes on its targets, which are critical for the nucleotide exchange activity, SopE utilizes different chemistry than Tiam1. This finding indicates that, remarkably, evolution has found more than one mechanism to execute essentially the same biochemical activity. Likewise, the crystal structure of SptP alone does not suggest that it would have GAP activity. However, when viewed in complex with its target, its GAP activity can be clearly inferred (Figure 1B) (Stebbins and Galán, 2000). In this case, however, SptP utilizes chemistry similar to that utilized by most eukaryotic cell GAPs, which involves the precise positioning of a critical arginine residue within the active site of the GTPase. Therefore, SopE and SptP can be considered "convergently evolved" mimics of their eukaryotic cell counterparts.

Other examples of mimicry to modulate Rho family GTPase function are even more subtle. For example, the *E. coli* T3SS effector EspF(U) mimics an autoinhibitory domain found within N-WASP (Cheng et al., 2008). Through this mimicry, EspF(U) activates N-WASP by competitively disrupting its autoinhibited state. In this sense, EspF(U) functionally mimics Cdc42, which activates N-WASP in a similar manner. Another example of subtle mimicry is a group of T3SS effectors, collectively known

Table 1. Examples of Mimicry in Type III Secretion Effectors

Effector protein	Bacteria	Mimicry	Phenotype	References
SopE	*Salmonella enterica*	GEF for Rho family GTPases	Bacterial entry into nonphagocytic cells	Hardt et al., 1998b
SptP (N terminus); YopE/ExoS (N terminus)	*Salmonella enterica*;*Yersinia* spp./ *Pseudomonas aeruginosa*	GAP for Rho family GTPases	Recovery of the actin cytoskeleton after bacterial internalization (SptP); disruption of the actin cytoskeleton (YopE and ExoS)	Fu and Galán, 1999; Von Pawel-Rammingen et al., 2000; Goehring et al., 1999
SopA	*Salmonella enterica*	HECT-like E3 ubiquitin ligase	Promotes inflammation	Diao et al., 2008
SseL	*Salmonella enterica*	Ubiquitin protease	Macrophage killing	Rytkönen et al., 2007
SspH and IpaH family members	*Salmonella enterica* and *Shigella* spp.	E3 ubiquitin ligase	Unknown	Rohde et al., 2007
SopB/IpgD	*Salmonella enterica* and *Shigella* spp.	Phosphoinositide phosphatases	Stimulation of SGEF, modulation of vesicular trafficking, Akt activation (SopB); PI3 Kinase activation (IpgB)	Patel and Galán, 2006; Hernandez et al., 2004; Steele-Mortimer et al., 2000
XopD (several family members)	*Xanthomonas campestris*	Cysteine protease for SUMO	Disruption of plant defense response	Hotson et al., 2003
IpgB/SifA and other family members	*Shigella* spp. and *Salmonella enterica*	Activated Rho family GTPases	Modulation of the actin cytoskeleton (IpgB); endosomal tubulation (SifA)	Alto et al., 2006; Ohlson et al., 2008
EspF/TccP2	*E. coli*	N-WASP autoinhibitory domain	Actin nucleation at the plasma membrane	Cheng et al., 2008
IpaA	*Shigella* spp.	Vinculin	Actin reorganization and bacterial internalization into nonphagocytic cells	Izard et al., 2006
AvrPtoB and family members	*Pseudomonas syringae*	RING-like E3 ubiquitin ligase	Inhibition of programmed cell death	Janjusevic et al., 2006
GALA family of proteins	*Ralstonia solanacearum*	F-box proteins	Promotes virulence	Angot et al., 2006
VopF	*Vibrio cholerae*	Formins/spire	Promotes actin nucleation	Tam et al., 2007

as the WxxxE family of effector proteins, which include the *Shigella* spp. effectors IpgB1 and IpgB2 and the *S.* Typhimurium effector SifA. These effectors trigger responses equivalent to those stimulated by activated Rho family GTPases by mimicking the activated state of these small G proteins, and recent structural studies reveal that although there is no primary sequence similarity, they are structural mimics of bacterial GEFs such as SopE (Alto et al., 2006; Ohlson et al., 2008).

Other examples of mimicry are seen in T3SS effectors that target the ubiquitination machinery. Protein ubiquitination is a multistep enzymatic process that results in the addition of ubiquitin to internal lysine residues of the substrate protein (Glickman and Ciechanover, 2002). The process involves an ubiquitin-activating enzyme (E1), which transfers ubiquitin to a family of ubiquitin-conjugating enzymes (E2s). Ubiquitin-loaded E2s are then recruited to their substrates by a family of ubiquitin ligases (E3s), which play a critical role in substrate recognition. Some T3SS effectors, exemplified by *S.* Typhimurium SopA or *Pseudomonas syringae* AvrPtoB, mimic the HECT- and RING-domain type of E3 ubiquitin ligases, respectively (Figure 1C) (Diao et al., 2008; Janjusevic et al., 2006). In contrast, a recently identified family of T3SS effector proteins with E3 ligase activity mimics the cysteine-based chemistry of HECT E3 ligases but shares no structural similarity to any known eukaryotic cell E3 ligases (Figure 1C) (Quezada et al., 2009; Singer et al., 2008; Zhu et al., 2008).

From the examples discussed here and others listed in Table 1, it is apparent that mimicry is emerging as one of the most common features in the function of T3SS effector proteins. In the context of the biology of most bacteria-encoding T3SSs, mimicry appears to be a very suitable strategy to interact with the host, because it usually leads to reversible modulation of cellular functions without overt harm to the target cell. Since this mimicry most often cannot be gleaned from the primary amino acid sequence or even from the *proto* crystal structures of the effectors, a number of bioinformatics or high-throughput crystallographic approaches are of limited use in the study of T3SS effector proteins. Rather, in most cases, the identification of their specific targets or even the solution of the atomic structures of the effector/target protein complexes is required to characterize their function.

Covalent Modifications of Host Cell Proteins

A number of effector proteins exert their function by introducing covalent modification to target cellular proteins (Table 2). In some instances, these covalent modifications are reversible and presumably aimed at modulating cellular functions by transiently altering the activity of the cellular targets. Phosphorylation

Table 2. Examples of Covalent Modifications Introduced by Type III Secretion Effectors

Effector protein	Bacteria	Covalent modification	Targets	References
YopH/SptP (C terminus)	*Yersinia* spp. and *Salmonella enterica*	Protein tyrosine phosphatase	Many	Guan and Dixon, 1990; Kaniga et al., 1996
YpkA/OspG	*Yersinia* spp. and *Shigella* spp.	Serine/threonine protein kinase	Gαq subunit of heterotrimeric G proteins (YpkA); ubiquitin-conjugating enzymes (OspG)	Navarro et al., 2007; Kim et al., 2005
YopJ/AvrA and other family members	*Yersinia* spp. and *Salmonella enterica*	Acetylation	MAP kinase kinases	Mukherjee et al., 2006; Jones et al., 2008
OspF/SpvC	*Shigella* spp. and *Salmonella enterica*	Phosphothreonine lyase	MAP kinases	Li et al., 2007
ExoS/ExoT (C terminus)1	*Pseudomonas aeruginosa*	ADP ribosylation	Many	Barbieri, 2000
VopS	*Vibrio parahaemolyticus*	AMPylation	Rho family GTPases	Yarbrough et al., 2009

is one of the most common mechanisms of signal transduction utilized by eukaryotic cells. It is therefore not surprising that several T3SS effectors target this process to modulate cell function. For example, serine/threonine protein kinases have been identified in *Shigella* spp. (e.g., OspG) (Kim et al., 2005) and *Yersinia* spp. (e.g., YpkA/YopO) (Galyov et al., 1993). The *Shigella* OspG effector phosphorylates host ubiquitin-conjugating enzymes to thwart innate immune responses (Kim et al., 2005). The *Yersinia* YpkA/YopO kinase phosphorylates Gαq, thus inhibiting G protein-coupled receptor signaling (Navarro et al., 2007), although the significance of this activity for *Yersinia* spp. pathogenesis is unknown. In addition, several effectors with protein phosphatase activity have also been identified. This includes the *Yersinia* YopH and *Salmonella* SptP effectors, which disrupt macrophage function and MAP kinase signaling, respectively, by targeting several tyrosine-phosphorylated cellular proteins (Bliska and Black, 1995; Murli et al., 2001).

More recently, several effector proteins with enzymatic activities resulting in unusual covalent modification have been discovered. For example, a family of T3SS effectors, which includes the *Shigella* spp. OspF and *Salmonella* SpvC proteins, was shown to inhibit MAP kinase signaling by an unusual post-translational modification. This modification involves a phosphothreonine lyase enzymatic activity, which results in the irreversible removal of the phosphate group from phosphothreonine and its conversion into dehydrobutyrine (Li et al., 2007). This activity has not yet been described in eukaryotic cells. Another example of an unusual covalent modification associated with an effector is the *Yersinia* YopJ protein, which inhibits various signal-transduction pathways by acetylating critical serine or threonine residues of a group of proteins belonging to the MAP kinase kinases (MKK) family (Mukherjee et al., 2006). Since the MKKs are activated by phosphorylation of the same residues targeted by YopJ, the modified kinases cannot be activated. Protein acetylation is a well-characterized regulatory mechanism of eukaryotic cell histones. However, histone acetyl transferases in eukaryotic cells modify protein function by acetylating lysine residues. Therefore, YopJ is the first identified enzyme that acetylates serine and threonine residues, and it has been suggested that YopJ may well mimic a yet-to-be-identified class of eukaryotic enzymes that may regulate signaling via acetylation. Another unusual covalent modification mediated by the T3SS effector protein VopS has been described in *Vibrio parahaemolyticus* (Yarbrough et al., 2009). This effector covalently modifies a conserved threonine residue on Rho, Rac, and Cdc42 by adding adenosine 5′-monophosphate (AMP), a process that has been referred to as "AMPylation." AMPylated Rho GTPases are unable to interact with downstream effectors, thereby inhibiting actin dynamics in the infected cell. Interestingly, AMPylated proteins have also been detected in eukaryotic proteins in the absence of bacterial infection. Although it is still unclear whether these modifications are reversible, the finding of acetylated and AMPylated proteins within the host cell suggests that this may well be the case and that these posttranslational modifications may be involved in cell signaling. These examples of novel activities associated with T3S effectors that may be present in eukaryotic cells showcase how the study of bacterial virulence factors may lead to important new insights into basic cell biology.

Work in Concert with Other Effector Proteins

One of the most daunting challenges in the study of T3SS effector proteins is the fact that they exert their function in the context of other effector proteins. Indeed, it can be argued that, in contrast to toxins (as defined above), T3SSs have evolved to modulate complex cellular functions in a manner that requires the coordinated activity of many effectors delivered in a precise temporal and spatial manner. For example, the *Salmonella* T3SS effector SptP is a GAP for several Rho-GTPase family members (Fu and Galán, 1999). Since one of the physiological roles of these small G proteins is to modulate actin dynamics, transient expression of SptP into mammalian cells leads to a profound change in the actin cytoskeleton. This observation led to the erroneous conclusion that the function of this effector was to disrupt the actin cytoskeleton, presumably to prevent phagocytosis (Fu and Galán, 1998). However, subsequent studies showed that the function of SptP was indeed the opposite. In the context of other *Salmonella* effectors that have the capacity to activate Rho family GTPases (e.g., the GEFs SopE and SopE2), the SptP GAP activity is required for the cells to recover homeostasis (Fu and Galán, 1999). Since profuse stimulation of Rho family GTPases by the bacterial effectors is harmful to the cell, *Salmonella* evolved an effector to put a "brake" to such stimulation and thus preserve the integrity of

the cell. This example clearly illustrates that the standard reductionist approaches commonly utilized in the field to study effector function (e.g., overexpression of single effectors within a cell) can lead to misinterpretation. To compound the challenge, T3SS effectors are usually delivered in very small amounts, which is in sharp contrast with many experimental approaches that result in a vast overexpression of a given effector within cells. Therefore, while the biochemical activities of many of T3SS effectors are known, the main challenge remains to identify their relevant cellular targets. In this regard, it will be essential to consider both the environment in which these effectors exert their function and the concentration that they achieve within the cell during infection. Once these parameters are considered, it is possible that some of the targets that have thus far been identified using standard approaches may prove to be irrelevant.

Similar Biochemical Activity in a Different Bacteria/Host Interface Results in Unique Effects

During the last few years, remarkable progress has been made in the identification of enzymatic activities associated with T3SS effector proteins, many of them discussed in this article. However, there has been much less progress in the understanding of the contribution of these activities to the host/pathogen interactions. Although the identification of the biochemical activity of a given effector is very helpful, it is certainly not sufficient to understand its potential role during infection. The identification of the physiological targets of those activities is essential to really understand effector function in the context of infection. As discussed above, T3SSs are central components of the host/pathogen interface. Given the great diversity in the pathogens that encode T3SS, the host/pathogen functional interface in which these systems participate varies considerably. Yet apparently homologous effectors (i.e., effectors with the same biochemical activities) are encoded by very different pathogens. The differences between host/pathogen functional interfaces must be considered before extrapolating findings from homologous effectors encoded by different pathogens, since it is possible that the same biochemical activity associated with such an effector may be directed to a different target or may result in a different effect. For example, some of these pathogens have evolved to gain access into host cells while others remain extracellular during their infection cycle. *Salmonella* spp. and *Yersinia* spp. are intracellular and extracellular pathogens, respectively, and yet they encode several apparently homologous effectors. *Yersinia* YopE and *Salmonella* SptP are both GAPs for Rho family GTPases (Fu and Galán, 1999; Von Pawel-Rammingen et al., 2000). Yet while YopE disrupts the actin cytoskeleton to prevent bacterial phagocytosis, SptP, as discussed above, counters the activity of other effectors to restore cellular homeostasis and the integrity of the actin cytoskeleton. The acetyl transferases YopJ and AvrA encoded by *Yersinia* spp. and *S.* Typhimurium are another example of apparently homologous effectors carrying out different functions. While YopJ broadly inhibits all MAP kinase pathways and NF-κB signaling by targeting all MKKs and IKKβ (Orth, 2002), AvrA, when delivered in physiological amounts into eukaryotic cells, only targets the Jnk pathway (Jones et al., 2008; F. Du and J.G., unpublished data). Therefore, it seems that evolution has shaped the activity of these highly related molecules to suit the functional interface of the respective pathogens that harbor them. In the case of *Yersinia* spp., an extracellular pathogen, YopJ has evolved to broadly inhibit the host innate immune response. In contrast, AvrA exerts a more subtle effect to presumably modulate but not prevent the transcriptional responses induced by *Salmonella*. The take-home message of this common theme is that the context in which a given effector exerts its activity is crucial for the understanding of its function during infection.

Precise Temporal Regulation

As discussed above, the function of different effectors delivered by the same pathogen is exerted in the context of the activities of other effectors, ultimately resulting in the modulation or induction of highly coordinated and complex cellular responses. Therefore, it is not surprising that the specific activity of individual effectors is highly regulated both temporally and spatially. There are several mechanisms involved in the temporal regulation of T3SS effector function. For example, although poorly understood at the mechanistic level, there is increasing evidence that there is a hierarchy in the engagement of T3SS effectors by the protein delivery machine (Collazo and Galán, 1996; Deng et al., 2005; Sorg et al., 2006; Wang et al., 2008). It is therefore possible that this mechanism may be central for the temporal coordination of the delivery of groups of effector proteins, particularly if these effectors must be delivered in a hierarchical fashion over a short period of time.

In addition, some pathogens encode more than one T3SS that are expressed at different times during infection and are able to recognize and deliver different effector proteins, whose expression is also temporally regulated. This may afford the pathogen the opportunity to temporally coordinate the delivery of groups of effector proteins that must exert their function at different times during infection. This is certainly the case in *Salmonella enterica*, which encodes two T3SSs in its pathogenicity islands 1 (SPI-1) and 2 (SPI-2) (Galán, 2001). The SPI-1 T3SS is expressed during *Salmonella*'s extracellular stage, while expression of SPI-2 is induced after internalization. Consequently, the SPI-1 T3SS mediates entry into cells (and the stimulation of transcriptional responses), while the SPI-2 mediates the sculpting of a specific intracellular niche that allows *Salmonella* to replicate. Each one of these T3SSs recognizes specific effector proteins, and consequently, it has been traditionally assumed that they exert their function in an independent manner. However, it is becoming increasingly clear that there is a much closer functional relationship between these two systems than originally thought. For example, in addition to mediating entry, the SPI-1 T3SS effector proteins divert the *S. enterica*-containing phagosome from being delivered into lysosomes (Hernandez et al., 2004; Steele-Mortimer et al., 2002). This in turn allows the expression of effectors of the SPI-2 T3SS, which further modulate vesicular trafficking to establish the final replicative niche. In addition, it is becoming clear that some of the T3SS effectors can be recognized by both T3SSs (Miao et al., 1999). Therefore, it appears that "hierarchy" alone may not be sufficient to temporally coordinate the delivery of many effectors that exert their function in a coordinate manner at different times over the infection cycle. Consequently, it seems that *Salmonella* has evolved

two separate T3SSs to be able to temporally coordinate the delivery of these effectors.

Yet another mechanism of temporal regulation involves the specific half-life of the effectors within the target cell. For example, as discussed above, the *Salmonella* T3SS effectors SopE and SptP possess opposite activities (GEF and GAP for the same Rho family GTPases, respectively), and yet they seem to be delivered into cells roughly at the same time. However, their half-lives within cells are quite different. While SopE is rapidly degraded by an ubiquitin-mediated process shortly after its delivery, SptP remains within cells for an extended period of time (Kubori and Galán, 2003). Finally, many bacterial pathogens encode E3 ubiquitin ligases, whose targets are unknown (Angot et al., 2007). It is possible that some of these enzymes may be involved in the temporal regulation of the activity of effector proteins delivered by the same T3SS.

Precise Localization and Functional Diversification

It is increasingly clear that effector proteins exert their function at precise locations within the cells, which is central to their function. Yet remarkably little is known about the mechanisms that restrict the location of an effector to a given compartment. This is, in part, due to the experimental difficulties in localizing effector proteins after translocation from the bacteria, since they are usually delivered in very low amounts. Furthermore, the restrictions in tag choice and position within the effector protein imposed by the protein delivery systems themselves have significantly hampered the development of reliable technologies to visualize in vivo the delivery and location of the effector proteins during infection. To add to the challenges, some effector proteins utilize the same domain to serve as signal for T3S from the bacteria and to serve as signal for proper localization within the eukaryotic host cell (Montagna et al., 2001). Some information on the localization of effectors within cells has been gleaned by transient expression of tagged forms of the effectors. However, as discussed above, these types of experiments may lead to artifacts and therefore should be interpreted with caution. In any case, through limited studies, two common themes are beginning to emerge in the mechanisms of effector protein localization.

One mechanism involves specific domains within the effector protein that serve to target the particular biochemical activity encoded in a different domain of the same effector (Rabin et al., 2006; Schlumberger et al., 2007; Zhang and Barbieri, 2005). This is a common mechanism that directs the localization and the activity of many host cellular proteins. Therefore, it is not surprising that many effectors have domains that share either primary amino acid sequence or structural similarity to domains known to target eukaryotic cell proteins. For example, a family of effector proteins encoded by different pathogens possesses a leucine-rich repeat domain that, at least in some cases, mediates the localization of their activity to specific locations (Benabdillah et al., 2004; Quezada et al., 2009). Leucine-rich repeat domains, which are in essence "protein-protein" interaction domains, often serve the same purpose in several eukaryotic cell proteins. In addition, some effectors possess nuclear localization signals, which allow them to utilize the nuclear import machinery to reach their final destination (Benabdillah et al., 2004; Szurek et al., 2002).

Another mechanism of effector localization involves their "posttranslocation" modification within the host cell. In this case, the effectors make use of cellular machinery to acquire additional information, which directs them to their specific site of action. For example, the localization of several T3SS effectors from the plant pathogen *Pseudomonas syringae* is mediated by consensus myristoylation sites, which upon myristoylation inside the host cell target these effectors to the plasma membrane (Nimchuk et al., 2000). In addition to serving as a signal for degradation, ubiquitination is also used as a targeting signal for some effector proteins. Such is the case of the *Salmonella* effector SopB, a phosphoinositide phosphatase which, with the same catalytic activity, mediates bacterial internalization (Zhou et al., 2001), stimulates the production of nitric oxide (Drecktrah et al., 2005), activates Akt (Steele-Mortimer et al., 2000), and modulates vesicular trafficking of the bacteria-containing vacuole (Hernandez et al., 2004; Mallo et al., 2008). Upon translocation from the bacteria, SopB is rapidly monoubiquitinated (Marcus et al., 2002), which results in its removal from the plasma membrane and its delivery to the *Salmonella*-containing vacuole (SCV) (Patel et al., 2009). In the absence of ubiquitination, SopB remains at the plasma membrane, where it continues to stimulate actin cytoskeleton rearrangements and Akt activation. However, nonubiquitinated SopB fails to localize to the SCV, resulting in defective intracellular bacterial growth (Patel et al., 2009). Thus, by exploiting the host ubiquitination machinery, *S.* Typhimurium not only properly localizes an effector protein but also broadens the functional repertoire of a virulence factor to maximize its ability to modulate cellular functions.

Functional Redundancy

A central element in the design of a robust system is to build functional redundancy. It is therefore not surprising that T3SSs have evolved quite a bit of redundancy, particularly regarding the activities of their effectors. This redundancy takes at least two forms. The simplest form is exemplified by the presence of highly related effectors with apparently similar function. There is, however, a different type of redundancy that is more subtle and consequently often difficult to identify. This type of redundancy involves effectors that have different biochemical activity but target a similar cellular process. For example, *Yersinia* spp. target Rho family GTPases to prevent phagocytosis through the activity of at least three different effectors, YopE, YopT, and YpkA (Trosky et al., 2008). However, although all of these effectors inhibit Rho family GTPases, they do so by different mechanisms. As discussed above, YopE is a GAP that potently inhibits several members of the Rho family. In contrast, YpkA inhibits Rho GTPases by mimicking host guanidine nucleotide dissociation inhibitors, thus inhibiting nucleotide exchange in these GTPases. On the other hand, YopT, a cysteine protease, exerts its inhibitory effect by removing the prenylated cysteine from the C terminus of the Rho GTPases, thus releasing them from membranes and preventing their function.

Another form of redundancy in the targeting of Rho GTPases is observed in *S.* Typhimurium. These bacteria stimulate Rho GTPases to mediate their own uptake into nonphagocytic cells. They do so through the functionally redundant activity of three T3SS effector proteins, SopE, SopE2, and SopB. As discussed above, SopE and SopE2 are GEFs for Rac1, Cdc42, and RhoG

(Hardt et al., 1998a; Stender et al., 2000). SopB, on the other hand, is a phosphoinositide phosphatase that activates Rho family GTPases by stimulating the activity of endogenous exchange factors through the metabolic fluxing of phosphoinositides (Patel and Galán, 2006). Naturally, the built-in redundancy in T3SSs adds to the difficulties in the study of the function of individual effector proteins, particularly when the redundancy is not immediately apparent. Indeed, it is a common occurrence that elimination of a single effector protein does not lead to a measurable phenotype, which hampers the study of the role of a specific effector during infection. The identification and elimination of all the redundant effectors is often required to be able to observe a phenotype. However, it is also likely that the lack of phenotypes often observed after the elimination of a single effector may not be due to redundancy, but rather to the lack of a specific assay capable of detecting the absence of such an effector. Therefore, the list of ''redundant effectors'' is likely to diminish as we develop more biological assays and learn more about these systems.

Concluding Remarks

The last few years have seen remarkable progress in the understanding of the function of bacterial effector proteins. This knowledge has already revealed common themes in the design and function of this remarkable family of bacterial virulence factors. The identification of the biochemical activities of many effector proteins has not only increased our understanding of these systems but has also potentially uncovered new basic cell biology. The challenge for the future remains gaining a better understanding of the contribution of the different bacterial effectors and the machines that deliver them to the biology and/or pathogenesis of the different bacteria that harbor them. This will necessitate the precise definition of the physiologically relevant targets of the different effectors, an area of research that has lagged behind because of the lack of suitable experimental tools or model systems. There is now firm evidence that indicates that effector proteins delivered by a given bacterium work in concert with one another. Therefore, the understanding of the function of a given protein delivery system as a whole will require the definition of the function of all or most of the effector proteins delivered by this particular system. Considering the fact that a given pathogen delivers multiple effector proteins, the task ahead is daunting but not less exciting. It is simply hoped that the next few years will be as exciting and productive as the last few.

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